Virus-like nanoparticle and DNA vaccination confers protection against respiratory syncytial virus by modulating innate and adaptive immune cells

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Abstract

Respiratory syncytial virus (RSV) is an important human pathogen. Expression of virus structural proteins produces self-assembled virus-like nanoparticles (VLP). We investigated immune phenotypes after RSV challenge of immunized mice with VLP containing RSV F and G glycoproteins mixed with F-DNA (FdFG VLP). In contrast to formalin-inactivated RSV (FIRSV) causing vaccination-associated eosinophilia, FdFG VLP immunization induced low bronchoalveolar cellularity, higher ratios of $CD11c^+$ versus $CD11b^+$ phenotypic cells and $CD8^+$ T versus $CD4^+$ T cells secreting interferon (IFN)-γ, T helper type-1 immune responses, and no sign of eosinophilia upon RSV challenge. Furthermore, RSV neutralizing activity, lung viral clearance, and histology results suggest that FdFG VLP can be comparable to live RSV in conferring protection against RSV and in preventing RSV disease. This study provides evidence that a

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combination of recombinant RSV VLP and plasmid DNA may have a potential anti-RSV prophylactic vaccine inducing balanced innate and adaptive immune responses.

Keywords

RSV; nanoparticle vaccine; prophylactic vaccine; bronchoalveolar cells

Background

The World Health Organization estimates that respiratory syncytial virus (RSV) causes 64 millions of infection and 160,000 deaths globally [1]. In the 1960s, a formalin-inactivated RSV vaccine (FI-RSV) induced exacerbated disease during the next winter season, resulting in 80% hospitalizations and two deaths [2,3]. Despite the extensive endeavor to develop RSV vaccines, there is no licensed vaccine. Purified RSV fusion (F) or attachment (G) glycoprotein subunit and recombinant vectored RSV vaccines are also known to cause enhanced RSV disease (ERD) [4-6]. RSV reinfection is common throughout life, indicating that natural RSV infection fails to establish long-lasting immunity [7,8].

Virus-like nanoparticles (VLP) can be generated through the assembly of structural proteins and lipid bilayer membranes, and are morphologically similar to the virus [9,10]. Recent studies reported that a chimeric Newcastle disease virus (NDV) core protein VLP containing the RSV F and G ectodomains was produced by DNA transfection of avian cells [11,12]. DNA vaccines encoding either RSV F or G developed IgG2a antibodies, T helper (Th) type 1/Th2 cytokine responses, but potential concerns of pulmonary disease remain controversial [13,14]. A combination of RSV F DNA and protein vaccines was shown to induce F-specific antibodies and cytotoxic $CD8^+$ T cell responses in a neonatal mouse model [15].

Recombinant baculovirus-derived VLP vaccines containing the full-length of RSV F or G in a membrane-anchored form are in a range of 60 to 120 nm particles and shown to induce immune responses contributing to clearing lung viral loads [16]. DNA vaccines induce host immune responses by transduced cells expressing protein antigens endogenously and the efficacy of DNA vaccines is less likely to be affected by maternal antibodies. Innate and adaptive immune responses contributing to RSV protection remain unknown. We hypothesized that a combination of DNA and VLP vaccines would develop balanced immune responses to ensure virus clearance but not to inflame tissues causing RSV lung disease. In comparison with FI-RSV and live RSV, we investigated bronchoalveolar cellular phenotypes after immunization with a combined vaccine of RSV F DNA and VLP containing RSV F and G glycoproteins (FdFG VLP).

Methods

Preparation of RSV VLPs, RSV F encoding DNA, and FI-RSV

Nanoparticle VLP consisting of an influenza virus matrix (M1) protein core and RSV glycoproteins F (RSV F VLP) or G (RSV G VLP) on the surface were produced using the insect cell expression system and characterized as described [16]. Briefly, SF9 insect cells were infected with recombinant baculoviruses expressing M1 and RSV F or RSV G

proteins, and RSV VLP nanoparticles released into cell culture media were purified by ultracentrifugation [16]. The plasmid DNA encoding RSV F protein (RSV F DNA) was previously described [17] and amplified in E. coli cells and purified using endotoxin-free kits (Qiagen). FI-RSV was prepared by formalin inactivation as described in the supplementary materials.

Immunization and challenge

Female BALB/c mice aged 6 to 8 weeks (Charles River) were immunized intramuscularly with RSV vaccines or infected intranasally with live RSV A2. Groups of mice (n=15) were intramuscularly immunized with RSV vaccines at week 0 and 4. For FI-RSV immunization, 2 μg and 1 μg of FI-RSV were used for prime and boost, respectively. A mixed RSV vaccine designated as FdFG VLP contained RSV F DNA, RSV F VLP and RSV G VLP. The priming dose of FdFG VLP was composed of 10 μg F VLP/10 μg G VLP/50 μg F DNA and the boosting dose was half of each component (5 μg F VLP/5 μg G VLP/25 μg F DNA). For a control group, mice (n=15) were intranasally infected with live RSV A2 strain (1×10^6 plaque forming units (PFU) for prime, 0.5×10^6 PFU for boost) at weeks 0 and 4. Serum samples were collected at 3 weeks after prime and boost immunization. At 26 weeks after boost immunization, naïve, immunized, and infected mice were challenged with RSV (1×10^6 PFU/mouse). Animal experiments in this study were performed under the approval of Georgia State University (GSU) IACUC review boards (IACUC A11026). We followed the guidelines of the GSU IACUC for all animal experiments and husbandry. GSU IACUC operates under the federal Animal Welfare Law and regulations of the Department of Health and Human Services.

Antibody ELISA and RSV neutralizing activity

To determine antigen-specific antibody levels, maxisorp immunoplates were coated with FI-RSV (4 μg/ml), RSV F protein (100 ng/ml, BEI, NIH) or RSV G protein (200 ng/ml, Sino biological Inc.) as an ELISA antigen. RSV-specific neutralizing antibody titers in mouse sera were measured using the red fluorescent monomeric Katushka 2 protein expressing RSV A2 strain (A2-K-line19F) [18,19] as detailed in the supplementary materials.

Lung and BALF samples, flow cytometry analysis, and determination of virus titers

Day 5 post RSV challenge, mice were euthanized to collect bronchoalveolar lavage (BAL) fluids (BALF) and lung samples. BALF samples were obtained by infusing 1 ml of phosphate buffered saline into the lungs via trachea using a 25-gauge catheter [20].

For cell phenotype analysis, the harvested BAL cells from BALF samples (n=5, pooled) were stained with fluorophore-labeled surface markers (CD45, CD11b, CD11c, CD3, CD8, Fc blockers). An antibody cocktail for cell surface staining and intracellular interferon-gamma (IFN- γ) staining were used as described in supplementary methods. The lung homogenates were centrifuged at 2000 rpm for 10 minutes to collect supernatants. The virus titer in supernatants was determined by an immunoplaque assay [21].

Results

A combined VLP and DNA vaccine induces high IgG2a/IgG1 antibody ratios

Both F VLP and G VLP were shown to raise similar RSV neutralizing titers and control lung viral loads [16]. In addition, antibody responses specific for RSV G central domains were demonstrated to contribute to conferring protection and ameliorating RSV disease [22]. We found that FdFG VLP was more effective in inducing higher levels of IgG2a antibodies (Th1 type) whereas F DNA alone was not highly immunogenic (Supplementary Fig. S1). Therefore, to further evaluate the protective immune responses and safety of FdFG VLP in comparison with FI-RSV and live RSV, mice were intramuscularly immunized with FdFGVLP, FI-RSV, or infected with live RSV (Fig. 1). At 3 weeks after prime and boost immunization, RSV specific serum antibodies were determined using FI-RSV as an ELISA coating antigen (Fig. 1A). Highest levels of IgG1 antibody were detected in the group of FI-RSV whereas FdFG VLP immunized mice showed lowest levels of IgG1 isotype antibody (Fig. 1A). The live RSV group showed a similar level of IgG1 and IgG2a antibodies specific for RSV after the 1st infection, which was significantly increased after the 2nd dose of infection (Fig. 1A, B). A higher level of IgG1 antibodies was induced in FI-RSV immunized mice compared to that in FdFG VLP immunized or live RSV infected mice (Fig. 1A, B). As a result, the FdFG VLP group showed the highest ratios of IgG2a/IgG1 in particular after prime immunization (Fig. 1B).

When ELISA was performed using the purified RSV F protein as a coating antigen, IgG1 antibodies were induced at higher levels by prime vaccination with FI-RSV than those by priming with FdFG VLP or infection with live RSV (Fig. 1C). In contrast, FdFG VLP immunization or live RSV infection induced higher levels of IgG2a antibodies specific for RSV F than those of IgG1. Consequently, higher ratios of IgG2a/IgG1 antibodies were induced in the FdFG VLP group, followed by live RSV infection (Fig. 1D).

FI-RSV immunization also induced significantly higher levels of RSV G protein specific IgG1 and IgG2a antibody responses than live RSV infection or FdFG VLP immunization after prime immunization (Fig. 1E). Because of low levels of IgG1 isotype antibodies, relatively higher ratios of IgG2a/IgG1 were observed in the FdFG group (Fig. 1F). The pattern and levels of antibody responses were maintained for over 6 months (data not shown). These results indicate that FdFG VLP vaccine induces IgG2a antibodies predominantly recognizing the RSV F protein antigen. In line with high levels of IgG2a antibody responses, IFN-γ was secreted at higher levels by stimulation of whole splenocytes with live RSV, F or G VLP than the level of IFN-γ with FIRSV (Supplementary Fig. S2).

Control of RSV replication by immunization with RSV vaccines

RSV neutralizing activity is considered an important protective immune correlate of RSV vaccines. To determine RSV neutralizing activity of immune sera, we performed neutralizing antibody titration by using the RSV A2 strain expressing red fluorescent monomeric Katushka 2 protein [18,19]. Immune sera from the FI-RSV, FdFG-VLP and live RSV immunized mouse groups showed significantly decreased levels of fluorescent intensity compared to naïve mouse sera, indicating the inhibition of RSV infection (Fig.

2A). Up to 200 dilutions, approximately 50% decrease in RSV replication were observed in immune sera and there were no statistical significances among different vaccine immune sera (Fig. 2A). Thus, sera from FI-RSV, FdFG VLP and live RSV immunized mice exhibited high levels of RSV neutralizing activities compared to naïve sera.

To determine whether immunization with FI-RSV or FdFG VLP could protect mice against RSV replication, immunized or previously infected mice were intranasally challenged with RSV (1×10^6 PFU/mouse) at 26 weeks of post boost immunization. RSV titers were analyzed in the lung samples collected at day 5 post RSV challenge (Fig. 2B). Unimmunized naïve mice that were infected with RSV showed the highest levels of lung viral loads. RSV was detected at significantly lower levels in the lungs from mice that were previously immunized with FI-RSV or FdFG VLP, or previously infected with RSV compared to those of naïve mice (Fig. 2B). Thus, mice that were immunized with FI-RSV or FdFG VLP controlled RSV replication in lungs after RSV challenge infection.

FdFG VLP immunization prevents severe cellular infiltration into airway upon RSV infection

Phenotypes of immune cells contributing to RSV disease or protection are not completely defined. We determined whether FdFG VLP immunization would reduce infiltrating cells into airway upon RSV infection compared to FI-RSV immunization (Fig. 3). Naïve mice without infection exhibited low percentages (data not shown) and cellularity of lymphocytes (Fig. 3B). At day 5 post RSV infection, naïve mice showed a moderate increase in lymphocytes and cellularity (Fig. 3A, B). As expected, FI-RSV immunized mice showed the highest cellular infiltrates with large size cell populations (Region 1 gate, Fig. 3) which include granulocytes, dendritic cells, and monocytes and macrophages in bronchoalveolar cells (Supplementary Table 1, Fig. 3B). FdFG VLP immunized mice showed a lower level of cellularity in bronchoalveolar cells compared to those in FI-RSV immunized mice (Fig. 3B). Live RSV group that was previously infected two times with RSV also caused substantial levels of lymphocytes and granular/myeloid cells, which is higher than those by FdFG VLP immunization (Fig. 3B). The region 1 gated BAL cells of FI-RSV immunized mice were found to be smaller in size than those in naïve, FdFG VLP, or live RSV mice (Fig. 3A) and most cells in the region 1 gate from the FIRSV group are likely to be eosinophils (Fig. 5). Mice with FdFG VLP immunization showed a similar pattern of region 1 large cell populations as RSV-reinfected mice in response to RSV challenge (Fig. 3B).

FdFG VLP immunization modulates innate CD11b$^+$ and CD11c$^+$ cells in BALF

A better understanding of cellular phenotypes in BALF following RSV vaccination would be informative for determining immune responses associated with protection or lung inflammatory disease. The FI-RSV group showed a higher level of myeloid marker CD11b$^+$ cells compared to FdFG VLP or live RSV at day 5 post RSV challenge (Fig. 4A). FdFG VLP immunization of mice induced a moderate range in levels of CD11b$^+$ cells similar to that in the live RSV group after RSV challenge. In contrast, CD11c$^+$ cells were found at higher levels in the FdFG VLP group compared to the FI-RSV group. That is, the FdFG VLP group exhibited a trend of increasing CD11c$^+$ cells and lowering CD11b$^+$ cells, which is similar to a pattern observed with the group of live RSV infection. As a result, the ratios

of CD11c$^+$ versus CD11b$^+$ cells were highest in the FdFG VLP group whereas FI-RSV showed the lowest level of CD11c$^+$ cells among the groups after RSV challenge (Fig. 4B, C). CD45$^+$CD11c$^+$ cells appeared to be alveolar macrophages with a F4/80$^+$ phenotype (data not shown) and the majority of CD45$^+$CD11b$^+$CD11c$^-$ cells seemed to be eosonophils (Fig. 5). These results suggest that an imbalance in CD11c$^+$ versus CD11b$^+$ cells and cell types in airway might be an important parameter contributing to FI-RSV vaccination-induced lung inflammatory disease with severe infiltrates around the airways and interstitial spaces (Supplementary Fig. S3).

FdFG VLP immunization does not induce eosinophilia upon RSV infection

Eosinophils were demonstrated to have the phenotypes of CD45$^+$CD11c$^-$CD11b$^+$SiglecF$^+$ and enriched in inflamed lung tissues [23,24]. At day 5 post RSV challenge, the FI-RSV immunized group induced prominently a population with CD11b$^+$SiglecF$^+$ cells, which was approximately 83% out of the CD45$^+$CD11c$^-$ large cell gated populations (Fig. 5A) and 45% out of total BALF cells (Fig. 5B). High levels of eosinophils showed a correlation with severe infiltrates around the bronchial airways and interstitial spaces from the lung histology of FI-RSV immune mice (Supplementary Fig. S3). A low but distinct population with CD45$^+$CD11c$^-$CD11b$^+$SiglecF$^+$ cells at a level of approximately 14% (Fig. 5A) was also observed in the live RSV group after RSV challenge. Importantly, the group of mice immunized with FdFG VLP vaccine did not show such a distinct population of CD11b$^+$SiglecF$^+$ cells. Unimmunized naïve mice also showed CD11b$^+$SiglecF$^+$ phenotypic cells even at a low level after RSV infection (Fig. 5). Therefore, results in this study provide evidence that FdFG VLP immunization would not induce pulmonary eosinophilia whereas FI-RSV immunization induces severe eosinophilia and live RSV re-infections may induce a low level of eosinophils.

FdFG VLP immunization increases the ratios of adaptive CD8$^+$/CD4$^+$ cells secreting IFN-γ in BAL lymphocytes

Previous studies demonstrated that FI-RSV immunized mice had an increased number of CD4$^+$ T cells infiltrating BALF after RSV challenge, which was shown to be involved in vaccine-enhanced lung disease [25]. Thus, it was assumed that a reverse trend would be beneficial in preventing lung disease. CD4$^+$ T cells and CD8$^+$ T cells in BALF were analyzed day 5 post RSV challenge (Fig. 6). FI-RSV immunization induced high CD4$^+$ T cells in BALF after RSV challenge. In contrast, FdFG VLP immunization resulted in approximately 3-fold lower CD4$^+$ cellularity in BALF compared to that induced by FI-RSV immunization or by live RSV infection (supplementary Table 1). Overall, the ratios of CD8$^+$/CD4$^+$ T cells were relatively higher in the FdFG VLP and live RSV group than those in the FI-RSV group (Fig. 6B).

It was demonstrated that IFN-γ can have both beneficial protective and detrimental systemic disease effects [6], indicating that IFN-γ response should be balanced to avoid disease after RSV challenge. Intracellular IFN-γ cytokine staining of cells in BALF was presented (Fig. 6). FI-RSV immunization induced highest levels of IFN-γ producing CD4$^+$ T cells (32.8% of total CD3$^+$ T cells, Fig. 6A) whereas the FdFG VLP group showed a relatively low level of IFN-γ$^+$ CD4$^+$ T cells (16.4%, Fig. 6A). Since the CD4$^+$ T cellularity in the FI-RSV group

showed 3 fold higher than that in the FdFG VLP group (supplementary Table 1), the total IFN-γ producing CD4$^+$ T cells induced by FI-RSV immunization were approximately 5- to 6-fold higher than those by FdFG VLP immunization. Interestingly, live RSV showed an intermediate level of IFN-γ secreting CD4$^+$ T cells (22.7%, Fig. 6A). In line with high levels of IFN-γ producing CD4$^+$ T cells and eosinophils, we found that FI-RSV and live RSV more strongly stimulated *in vitro* bone marrow derived dendritic cells (BMDCs) to secrete proinflammatory cytokines IL-6 and TNF-α than RSV F or G VLP (Supplementary Fig. S2).

CD8$^+$ T cells were reported more likely to contribute to protection against RSV [26]. Contrary to IFN-γ^+ CD4$^+$ T cells, a reverse pattern of IFN-γ producing CD8$^+$ T cells between FI-RSV and FdFG VLP groups was observed in T cells from BALF after RSV challenge. The FdFG VLP group showed the highest level of IFN-γ^+ CD8$^+$ T cells (26.8% of total CD3$^+$ T cells, Fig. 6A, C). FI-RSV immunization induced a lowest level of IFN-γ^+ CD8$^+$ T cells (11.7%, Fig. 6A, C). Interestingly, FI-RSV was not effective in IL-12 Th1 type cytokine production from *in vitro* BMDC cultures (Supplementary Fig. S2). An intermediate level of IFN-γ^+ CD8$^+$ T cells was observed in the live RSV group (19.9%, Fig. 6A, C). Accordingly, the ratios of CD8$^+$ and CD4$^+$

T cells producing IFN-γ were highest in the FdFG VLP group (Fig. 6C), indicating that FdFG VLP immunization can modulate IFN-γ secreting CD4$^+$ and CD8$^+$ T cells infiltrating into airway upon RSV challenge. To further determine whether FdFG VLP could modulate the expression of cytokines in lung microenvironment, we determined IL-4, IL-5, IL-13 Th2 type and IFN-γ Th1 type cytokines in BALF as well as in lung extract (Supplementary Fig. S4) after RSV challenge of immunized mice. Lung extracts from FI-RSV immunized mice showed a trend of increasing Th2 cytokines (IL-4, IL-5, IL-13) whereas FdFG VLP immunization resulted in an increase of IFN-γ production in lung milieu. A similar pattern of cytokines was observed in BALF samples (Supplementary Fig. S4). Therefore, a pattern of increasing Th2 cytokines in lungs of FI-RSV immune mice might have contributed to inflammatory RSV disease.

Discussion

Protective immune correlates are not well understood because there is no licensed RSV vaccine. In particular, cellular phenotypes contributing to protection and disease remain largely unknown after RSV vaccination. Results in this study provide evidence that FdFG VLP could confer protection against RSV by preventing pulmonary eosinophilia and modulating cellular phenotypes as well as cellularity of infiltrates and IFN-γ secreting cells in addition to inducing Th1 type antibodies and cytokines.

FdFG VLP vaccination induced antibodies recognizing RSV, predominantly binding to the RSV F protein antigen and little to the RSV G antigen. After prime immunization with FdFG VLP, higher levels of IgG2a antibodies for RSV F were observed than those for RSV G (Fig. 1C and E), indicating that RSV F is more immunogenic than RSV G and this result is consistent with those in mice that were immunized with NDV VLPs containing both RSV F and G proteins [11]. After boost immunization, IgG2a antibodies for RSV G were increased. Meanwhile, IgG1 antibodies specific for RSV F were relatively increased after boost

immunization. Accordingly, IgG2a/IgG1 ratios showed an opposite direction between RSV F and RSV G specific antibodies after boost immunization (Fig. 1D, F). Therefore, antibody isotype profiles and distribution between RSV F and G specific antibodies may reflect an intrinsic difference in immunogenicity and protection.

RSV F is known to be an agonist for Toll-like receptor 4 (TLR4) [27]. There seems to be a certain correlation between TLR4 polymorphism and RSV disease severity [28]. TLR is known to regulate host immune responses against RSV [29]. High levels of IgG2a antibodies to RSV F than those to RSV G might be due to an effective stimulation of dendritic cells via TLR4 by F VLP. RSV neutralizing monoclonal antibodies targeting the RSV F protein have been licensed, making the F protein an attractive vaccine target [30]. Thus, it might be desirable that FdFG VLP immunization induced antibody immune responses that are predominantly specific for RSV F. Immune responses to RSV G were shown to be effective in controlling lung viral loads [16,12], and also to cause eosinophilia and secrete Th2 cytokines[31,32]. Also, purified RSV F protein vaccine was shown to induce a Th2-like response[33]. Higher levels of IgG2a antibodies were induced by immunization with FdFG VLP. Therefore, it should be informative to determine the contributions of each RSV FdFG VLP vaccine component to RSV protection and disease. Inclusion of F DNA in the FG VLP was found to contribute to further increasing IgG2a antibody responses (Supplementary Fig. S1). An enhanced Th1-like response induced by FdFG VLP might be due to the endogenous expression of genetic RSV F DNA vaccine plus intrinsic property of a nano-particulate nature of VLP. In support of this property of VLPs, influenza hemagglutinin proteins presented on VLP induced strong IgG2a isotype and IFN-γ producing T cell responses compared to soluble hemagglutinin proteins[20] .

There are some controversies regarding the efficacy of lung viral clearance in FI-RSV immunized animals. Low RSV neutralizing antibodies were reported to be induced by FI-RSV immunization[12,34,35]. Accordingly, low efficacy of lung viral clearance was shown in FI-RSV immunized mice[12,35]. In contrast, other previous studies demonstrated that FI-RSV immunized mice or cotton rats controlled RSV lung viral loads after infection[36-40]. It is not clear yet why lung viral clearance efficacies and RSV neutralizing titers are various among different studies on FI-RSV immunizations in animal models. FI-RSV preparation, doses of FI-RSV vaccines, animal models, and assay methods may influence the outcomes of FI-RSV vaccination efficacy despite its observed histopathology.

In this study, the total cell numbers of granulocyte/myeloid large cell populations and lymphocytes infiltrating BALF were found to be highest in the FI-RSV group. In contrast, FdFG VLP immunization did not result in significant infiltrates of leukocytes into BALF, and their bronchoalveolar cellularity was significantly lower than that by FI-RSV immunization following subsequent RSV infection. The group of mice intramuscularly immunized with FdFG VLP showed lower levels of granulocyte/myeloid cells and lymphocytes infiltrated into BALF upon RSV challenge even compared to those observed in the group of live RSV. Therefore, based on the results in this study, FdFG VLP is less likely to induce inflammatory cellular infiltrates into lungs upon RSV challenge compared to live RSV infection even at 26 weeks after immunization. FI-RSV immunization resulted in granulocyte/myeloid cells (region 1 gated cells in Fig. 3) that were smaller in size as shown

by forward light scattering, and the majority (~80%) of this region 1 gated granulocyte/ myeloid cell population was found to have eosinophil phenotypic markers ($CD45^+CD11c^-CD11b^+SiglecF^+$). The marked increase in pulmonary eosinophilia is a hallmark of FI-RSV vaccine-enhanced disease[3,41]. Also, the ratios of $CD11c^+$ cell phenotypes and $CD11b^+$ myeloid cell phenotypes ($CD11c^+/CD11b^+$) were very low in the FI-RSV group. Whereas, the FdFG VLP and live RSV groups of mice showed larger in size but low numbers of BALF granulocyte/myeloid cells that were composed of high levels of $CD11c^+$ phenotypic cells, and low levels of eosinophils. The FdFG VLP group showed even a lower level of eosinophils when compared to that in the live RSV group. The majority of $CD11c^+$ phenotypic cells in mice with FdFG VLP vaccine appeared to be alveolar macrophages with a $F4/80^+$ phenotype (data not shown) but further detailed studies should be carried out to define these cell types. The $CD11c^+$ phenotypic cells were shown to play a crucial role in inducing Th1-polarized adaptive immune responses [42,43]. $CD11b^+$ phenotypic cells were reported to promote the recruitment of leukocytes by pro-inflammatory cytokines following infection [44,45]. The high levels of $CD11b^+$ cells in BALF may be correlated with a property of FI-RSV in stimulating BMDCs to secrete IL-6 and TNF-α cytokines but not IL-12 or IFN-γ cytokines (Supplementary Fig. S1). In contrast, F VLP was less effective in stimulating BMDCs to secrete IL-6 and TNF-α cytokines. We observed a similar pattern of cellular phenotypes in lungs but less prominent compared to those in BALF (data not shown). Further characterization of these infiltrating cells in BALF and lungs after vaccination and RSV challenge will provide an informative insight into designing a safer vaccine against RSV. The results in this study suggest that modulation of innate immune cells in BALF by FdFG VLP vaccination plays an important role in conferring protection against RSV eosinophilic inflammatory disease.

T cells are known to contribute to RSV disease as well as protection, indicating that a balance between $CD4^+$ T cells and $CD8^+$ T cells is important[25,46]. It is highly significant to note that, among groups, FdFG VLP immunized mice showed highest levels of BALF $CD8^+$ T cells producing IFN-γ, which resulted in the highest ratio of $CD8^+/CD4^+$ T cells making IFN-γ. In contrast, FI-RSV mice exhibited highest levels of BALF $CD4^+$ T cells producing IFN-γ, giving the lowest ratio of $CD8^+/CD4^+$ T cells making IFN-γ. Live RSV mice showed over 2 fold less ratio of $CD8^+/CD4^+$ T cells making IFN-γ compared to those in FdFG VLP immunized mice. We also found that the cellularity of BALF $CD4^+$ T cells was highest in FI-RSV mice and then followed by live RSV mice whereas FdFG VLP mice showed relatively a low level of $CD4^+$ T cellularity in BALF. In addition to total BALF cellularity, high levels of IL-4 secreting lung and spleen cells were detected in FI-RSV immunized mice but not in FdFG VLP immunized mice (data not shown). In support of results in this study, IFN-γ producing $CD8^+$ T cells were shown to inhibit Th2 responses and pulmonary RSV disease[47]. Histopathology results of lung tissue sections suggest that FI-RSV immunization induced severe pulmonary inflammation, and moderate lung inflammation was observed with live RSV re-infections and unimmunized mice upon RSV infection (Supplementary Fig. S3). In contrast, FdFG VLP immunization did not induce such pulmonary inflammatory disease (Supplementary Fig. S3). Therefore, in addition to high cellularity in BALF, high levels of eosinophils, IL-4 and IFN-γ producing $CD4^+$ T cells, and Th2 cytokines may be all

together contributing to RSV lung disease upon infection, resulting in severe pulmonary histopathology.

In summary, we showed that FdFG VLP nanoparticulate vaccine could provide protection against RSV without causing eosinophilia. Phenotypic analysis of BALF cells suggested that FdFG VLP vaccination induced apparently balanced immune responses of CD11c^{+} phenotypic cells and IFN-γ producing CD8^{+} T cells locally. Also, high levels of CD11b^{+} eosinophils and IFN-γ producing CD4 appear to contribute to FI-RSV vaccination-induced pulmonary RSV disease. These results provide first evidence that RSV vaccines based on VLP in combination with F DNA genetic vaccine can be developed as an effective and safe RSV vaccine inducing protective immunity comparable or better than live RSV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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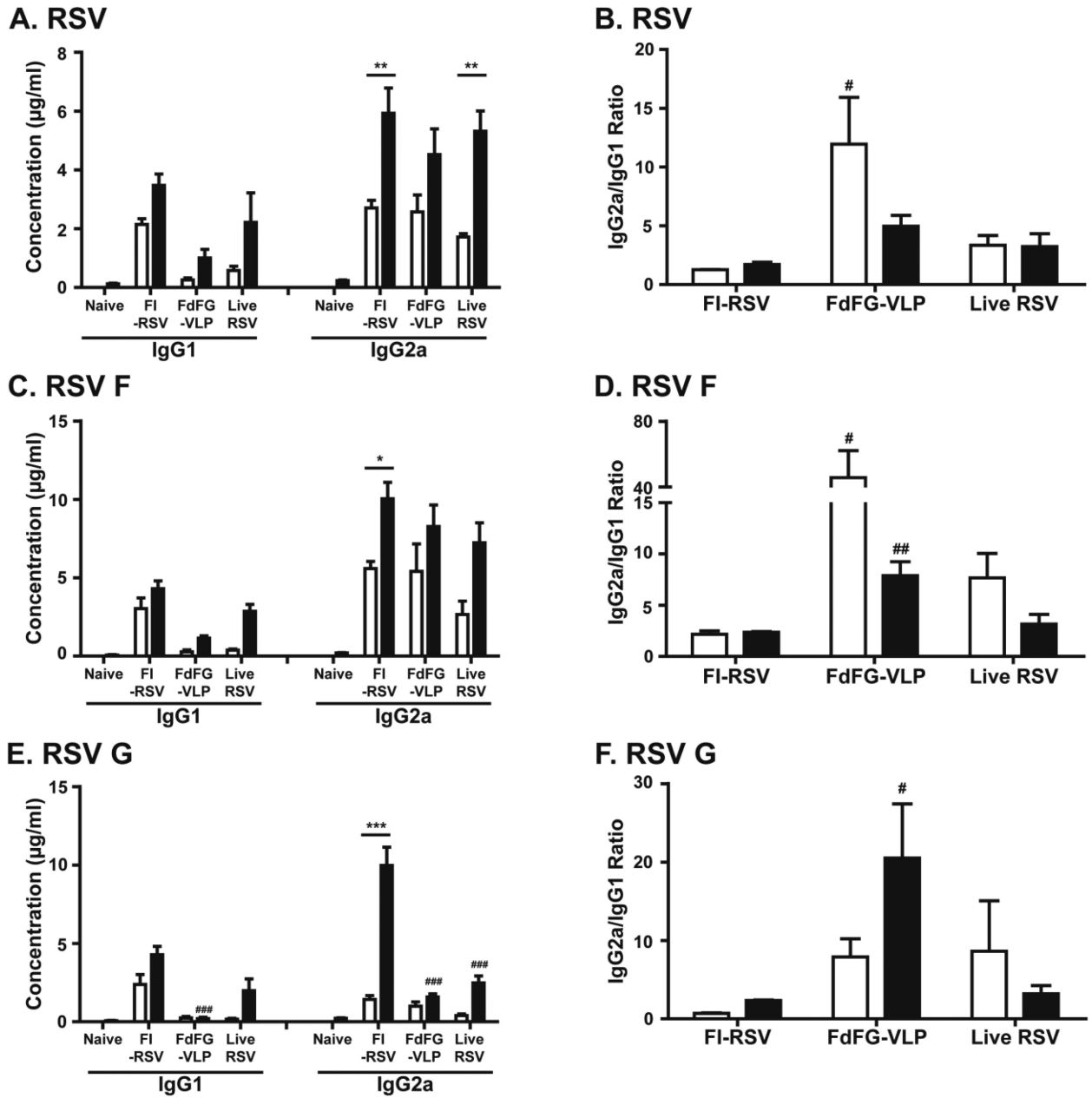
Figure 1. RSV-specific serum IgG isotype antibodies

(A) RSV specific IgG1 and IgG2a antibodies. (B) Ratios of IgG2a/IgG1 antibodies specific to RSV. (C) IgG1 and IgG2a isotype antibodies specific for purified RSV F protein. (D) Ratios of IgG2a/IgG1 isotype antibodies specific for RSV F protein. (E) IgG1 and IgG2a isotype antibodies specific for purified RSV G protein. (F) Ratios of IgG2a/IgG1 isotype antibodies specific for RSV G protein. * and *** indicates $p<0.05$ and 0.001, respectively, between prime and boost immunized sera. #, ## and ### mean $p<0.05$, 0.01 and 0.001, respectively, between the FI-RSV and FdFG VLP groups. Naïve: unimmunized mice. FI-RSV: FI-RSV vaccine. FdFGVLP: a combined vaccine of RSV F DNA, RSV F and G VLP. Live RSV: live RSV A2 strain.
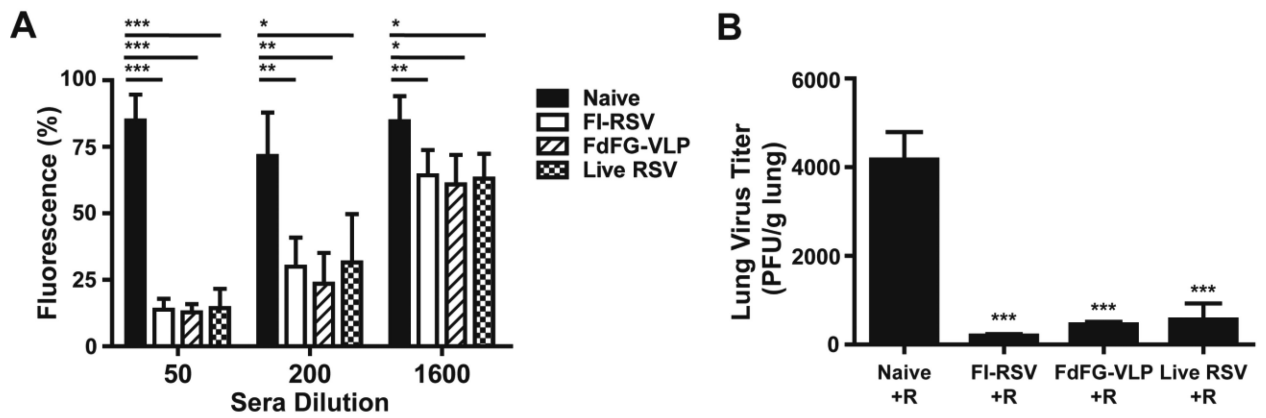
Figure 2. FdFG VLP immunization induces RSV neutralizing activity and controls lung viral loads

(A) RSV neutralizing activity in immunized sera. Data were presented as mean fluorescence percentages ± SEM. *, ** and *** indicates $p < 0.05$, 0.01 and 0.001, respectively, by student t test. (B) Lung RSV titers from naïve and immunized mice after RSV challenge. The results were representative out of 3 independent experiments. *** indicates $p < 0.001$ compared to the Naïve+R group.
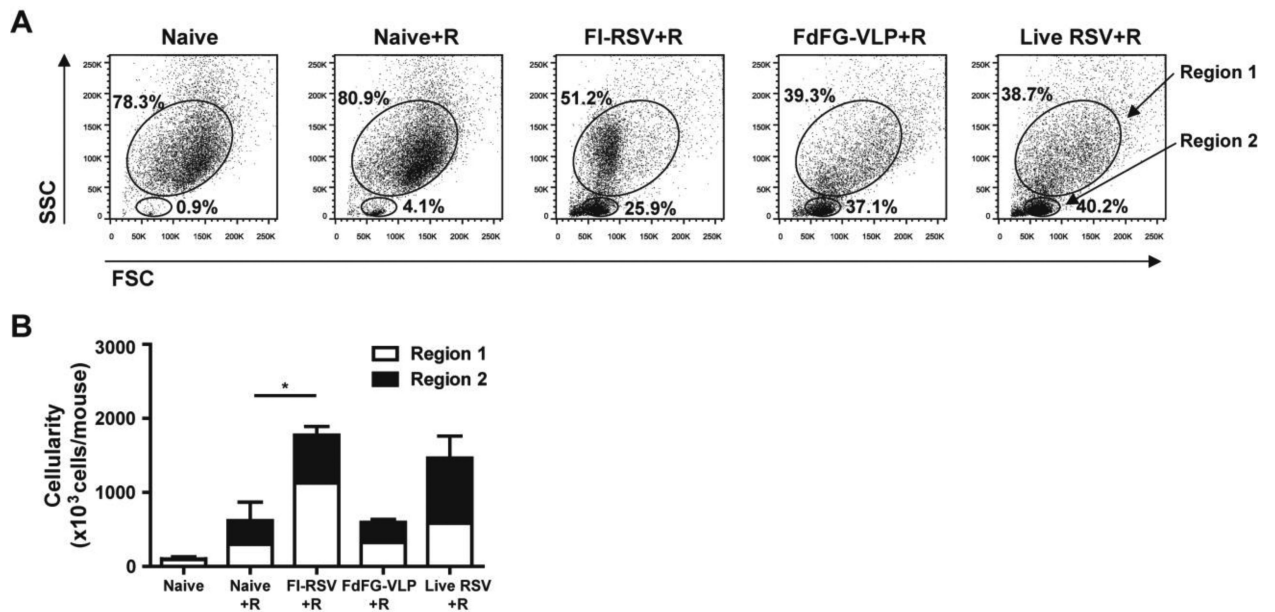
Figure 3. FdFG VLP immunization lowers bronchoalveolar cellularity compared to FIRSV or live RSV

(A) Flow cytometry profiles of BAL cells based on forward (size) and side (granularity) scattering. (B) Cellularity of Region 1 and 2 in BAL fluids. Cellularity was presented from the results of total BAL cell numbers per mouse multiplied by percentages of each population. The data are presented as mean ± SEM. * indicates p<0.05.
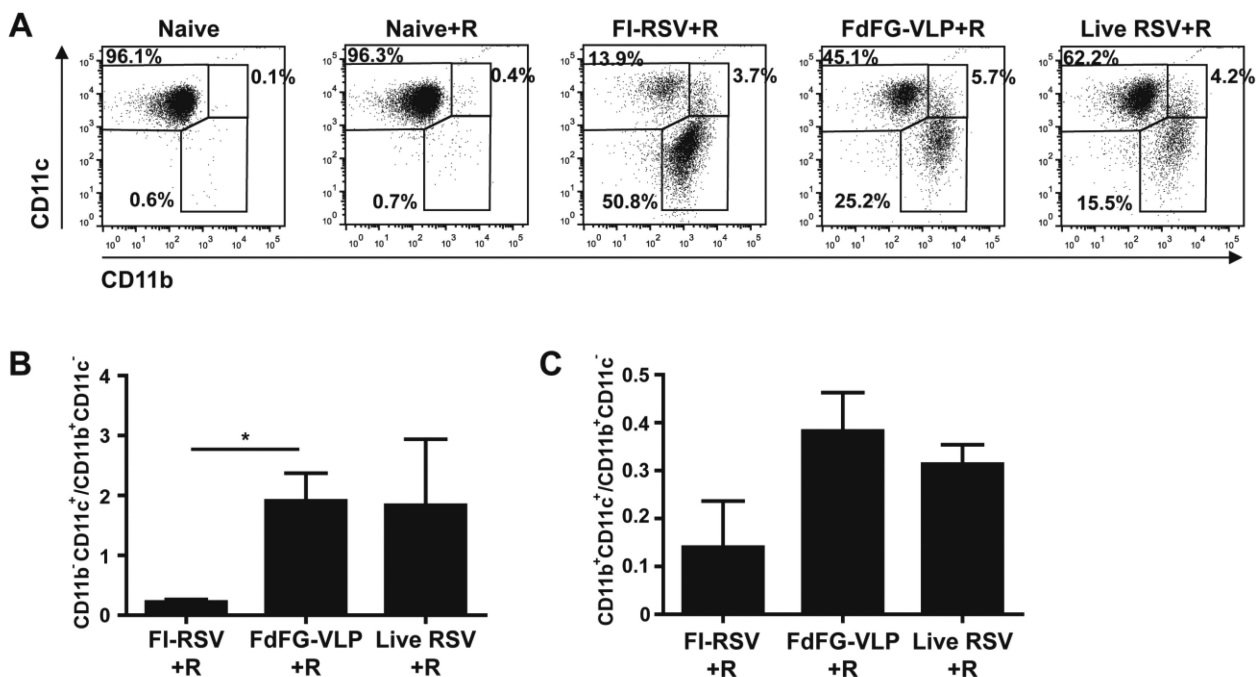
Figure 4. Distribution of CD11b and CD11c positive cells in bronchoalveolar lavage fluids
(A) Flow cytometry profiles gated on CD11b and CD11c. $CD45^+$ granulocyte/myeloid cells in BAL were gated by CD11b and CD11c expression. (B) $CD11b^-CD11c^+$ (upper-left in A)/ $CD11b^+CD11c^-$ (lower-right in A) ratios. (C) $CD11b^+CD11c^+$ (upper-right in A)/ $CD11b^+CD11c^-$ (lower-right in A) ratios. The ratios are presented as mean ± SEM. * indicates p<0.05 by student t test.

A. Eosinophils in large cell gates
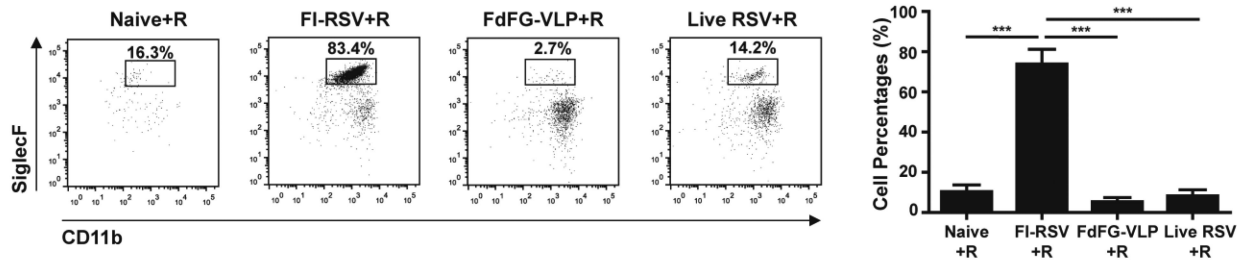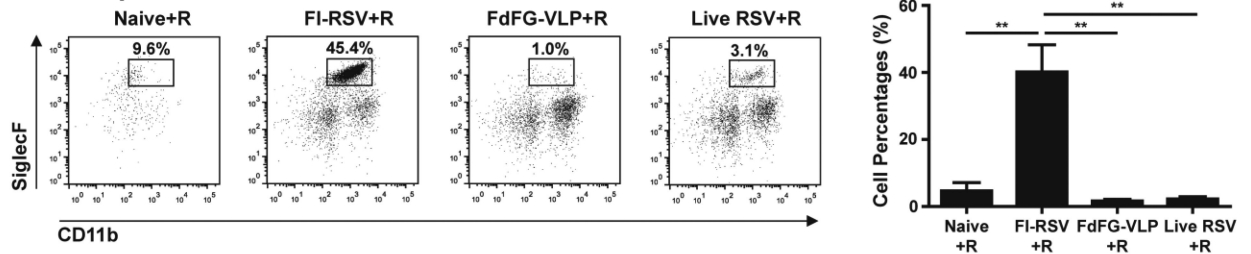

B. Eosinophils in total BAL cells

Figure 5. FdFG VLP immunization does not induce eosinophilia upon RSV challenge
(A) Eosinophils (CD11b$^+$SiglecF$^+$) in CD45$^+$CD11c$^−$ large cell gates of BAL cells. (B) Eosinophils (CD11b$^+$SiglecF$^+$) in CD45$^+$CD11c$^−$ total BAL cells. The mean percentage data are presented in right panels as mean ± SEM. *, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively.
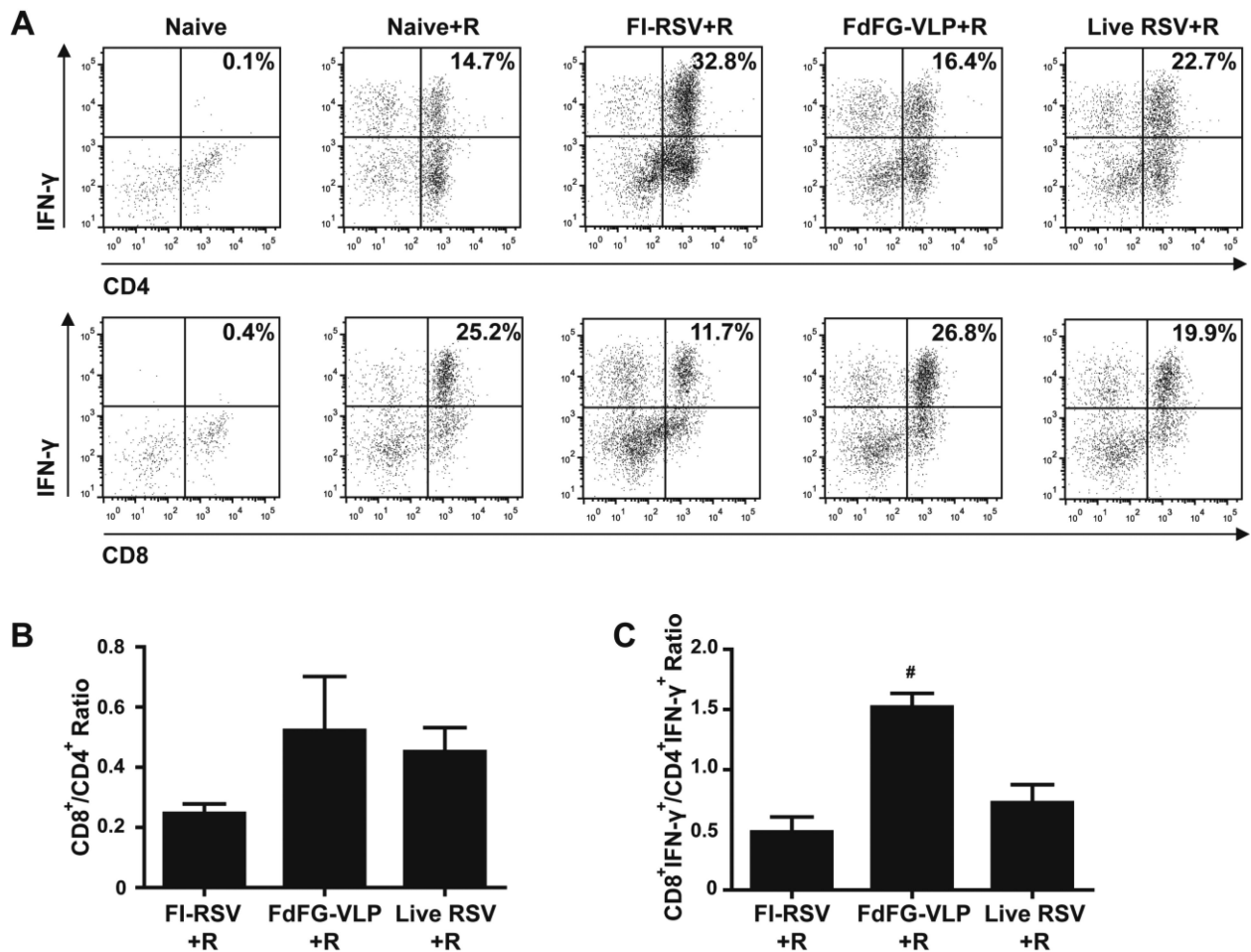
Figure 6. IFN-γ producing lymphocytes in bronchoalveolar lavage fluids
(A) IFN-γ$^+$ cell percentages in CD4$^+$ and CD8$^+$ lymphocyte (CD3$^+$) populations. The dot plots are representative of three independent intracellular cytokine staining experiments. (B) Ratios of CD8$^+$ to CD4$^+$ T cells in total lymphocyte population. (C) Ratios of IFN-γ producing CD8$^+$ to CD4$^+$ T cells in BAL lymphocytes. Ratios are presented as mean ± SEM. $^\#$ indicates p<0.05 compared to the FI-RSV+R group.